ISOLATION OF *BACILLUS SPHAERICUS* AND RELATED FORMS PATHOGENIC TO *CULEX QUINQUEFASCIATUS*

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ABSTRACT

Five hundred and forty nine isolates of *Bacillus spp.* sporeforming bacteria were obtained from soil, water, and mosquito larval samples collected in various localities of Yogyakarta Special Territory, Central Java and East Java. Four isolates were toxic to *Culex quinquefasciatus*; they belong to the species of *Bacillus sphaericus* (ascession number 23A and 51C), *Bacillus cereus* (ascession number 142A), and *Bacillus pumilus* (ascession number 25C).

INTRODUCTION

The adverse effects of chemical pesticides on human, domestic animals, plants, and wild life, and hence the need for environmental compatible pesticides in integrated pest management systems has increased the development of these systems and the use of microbial agents as insecticides. Among the microbial, sporeforming bacteria of the genus *Bacillus* have been the most popular for biological control of insect pests (including disease vectors) because: 1. Mass production by submerged fermentation is economical. 2. The endospore survives well in nature, 3. Members of this group are specific, innocuous in the environment and mainly atoxic to mammals[1].

The toxicity of *Bacillus thuringiensis* to Lepidoptera has been extensively investigated, and formulation based on this pathogen have long been commercialized. These products are increasingly applied to the practical control of pests harmful to agriculture and forestry. The same activities and interests are also true for public health entomology for almost three decades[1,2]. The potentialities of bacteria in controlling medically important insects have continuously been reported by many workers. To elucidate, it is important to note the isolation of *Bacillus thuringiensis* strain[3] designated subspecies israelensis (serotopy H-14) which was rapidly followed by the high selectivity of B.t.i. for a narrow range of Diptera, particularly mosquitoes and blackflies.

The rapid recognition of the potentialities of B.t.i. for the biocontrol of vectors was immediately followed by its commercialization for practical utilization in integrated control schemes. At the same time,

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several workers also reported the accurance of several other flagellar (H) serotypes of *B. thuringiensis* and *B. sphaericus* exhibiting toxicity to mosquitoes[4,5,6,7,8,9]

In viewing the worldwide distribution of naturally occuring sporeforming bacteria of the genus *Bacillus* which have been demonstrated by many workers, it is believed that a continuing search for such bacilli, in this case particularly in Indonesia, might well add highly effective "microbial insecticides" other than those commercialized (B.t.) to the armoury of medical entomologists. This paper describes the isolation and identification of several bacilli pathogenic to mosquitoes in Yogyakarta Special Territory, Central Java, and East Java.

MATERIALS AND METHODS

Isolation of Sporeforming Bacteria

Collection of samples was conducted in October 1988 from different parts of Yogyakarta Special Territory, Central Java, and East Java as shown in Table 1. Sample materials consisted of soil, mosquito larvae, and water. The first two materials were taken from drying ponds, creecks, canals, crop fields and river banks, while the water sample materials were taken from drying or confined breeding places of mosquitoes.

Table 1. Number of samples collected from different parts of Yogyakarta Special Territory, Central Java, and East Java.

Location	Type and number of samples			
	Larvae/Nos	Soil	Water	Total
Yogyakarta Spec.Ter.				
Yogyakarta	1 (43)	2	2	5
Bantul	1 (65)	1	1	3
Kulon Progo	2 (31)	6	6	14
Central Java				
Purworejo	5 (61)	17	15	37
Cilacap	1 (24)	3	3	7
Jepara	4 (512)	10	10	24
Semarang	2 (54)	22	22	46
Klaten	2 (41)	1	1	4
Magelang	5 (623)	4	4	13
East Java				
Merubetiri (Sukamade)	2 (26)	26	22	50
Total	25 (2713)	92	86	203

Isolation of *Bacillus spp* from soil samples was based on the method of Aizawa et al.[10] One gram of the sample was suspended in 10 ml of sterile distilled water and shaken vigorously for five minutes. After allowing the suspension to stand for ten minutes, the upper layer of the suspended sample was transferred to a test tube and heated at 65^0 for 30 min. Tenfold serial dilutions of the heated suspension in sterille distilled water or physiological saline (0,85 % NaCl) were plated on nutrient agar (pH 7.4). After incubation for two days at 28^0C *Bacillus* colonies were observed for determining characteristics of the colonies, while for observation of fully developed sporangium and parasporal inclusions the cultures were incubated for three to five days.

Isolation of *Bacillus spp* from larval samples was done as follows. Each sample was transferred to a test tube containing enough sterile saline and then homogenized. The homogenized suspension can be directly cultured or firstly heated as follows. One to two ml of the homogenized suspension was heated at 65^0C for 30 min. or at 80^0C for five min. After that the rest of the procedure was similar to the isolation of *Bacillus* from soil mentioned before.

To isolate *Bacillus* spp from water samples the water samples have to be centrifugated at 3000 rpm for 15 min. The next steps are the same as the procedures for isolation of *Bacillus* sporeformers from soil.

Qualitative Toxicity Test

The bacteria used for the toxicity test were grown on nutrient agar (pH 7.4) for seven to 10 days at 27-28^0C, then harvested and suspended in physiological saline. The suspension was centrifugated at 3000 rpm for 15 min. and washed twice. Tenfold dilutions of the bacterial sediment in physiological saline were made to obtain a concentration of 10^{-2} containing approximately 1.7-2.5 x 10^6 spores/ml for the toxicity tests.

In the toxicity test using *Culex quinquefasciatus,* 20 early L4 larvae were introduced into a petridish containing 40 ml seasonal water with 1 ml of the prepared bacterial suspension. Each treatment was replicated three times and the larvae reared at room temperature for 24 to 48 hours. Corresponding controls without bacterial culture were included in the tests.

RESULTS

The amount of the sample materials collected from different parts of the sampling area was 203, consisting of 25 larval samples, 92 soil samples, and 86 water samples as shown in Table 1. From the 203 samples there were 549 isolates of *Bacillus* spp obtained, among which 77 isolates from larval samples, and 236 isolates from each of the soil and water samples (Table 2).

Out of the 549 isolates, only four isolates were found to be toxic to *C.quinquefasciatus.* These were with the code numbers: 23A (from water, Pituruh-Purworejo), 25C (from water, Pituruh-Purworejo), 51C (from soil, Sewon-Bantul), and 142A (from soil, Merubetiri-Sukamade East Java), each of which was identified consecutively as 23A *B.sphaericus,* 25C *B.fumilus,* 51C *B.sphaericus,* and 142A *B.cereus* (by Dr.H. de Barjac, by letter). The toxicity of the isolates was examined qualitatively, and judging the mortalities resulted in the tests, the isolates were regarded as potential killers of the mosquitoes (Table 3).

Table 2. Number of isolates of sporeforming bacteria in Yogyakarta Special Territory, Central Java, and East Java

Location	Number of isolates			Total
	Larvae	Soil	Water	
Yogyakarta Spec.Ter.				
Yogyakarta	3	7	6	16
Bantul	2	5	6	13
Kulon Progo	9	20	20	49
Central Java				
Purworejo	12	44	36	92
Cilacap	0	7	10	17
Jepara	12	26	25	63
Semarang	0	48	57	105
Klaten	9	0	0	9
Magelang	25	13	14	52
East Java				
Merubetiri (Sukamade)	5	66	62	133
Total	77	236	236	549

Table 3. Average mortality of larvae due to application of different isolates of sporeforming bacteria (spores approximately 1.7-2.5 x 10^6/ml)

Isolate code nos	% Mortality (48 hr)	Type of sample	Location	Species
23 A	65	Water	River bank, Pituruh Purworejo	B.sphaericus (new strain*)
25 C	60	do	do	B.fumilus
51 C	55	Soil	Sewon, Bantul	B.sphaericus (new strain*)
142 A	62.5	do	marsh, Merubetiri (Sukamade)	B.cereus

*) according to Dr. H. de Barjac, Institut Pasteur, Paris

In the analysis of the characteristics of colony and cell morphology (Table 4), based on the grouping analysis of Parry et al (1983)[11] the isolates 25C and 142A belong to Bacillus group I, while the isolates 23A and 51C belong to group III.

Table 4. Characteristics of colony and cell morphology of different isolates of spore forming bacteria pathogenic to *C.quinquefasciatus*

Isolate code nos	Characteristics of Bacillus	
	Cell	Colony
23 A	rod shaped; spore; sphaerical, subterminal, slightly swallen; gram +	circular, convex, edge slightly smooth, greyish/pale, thick, diam. 3 - 4 mm.
25 C	rod shaped; spore; ellipsoidal, central, not swallen, gram +	circular, convex, edge rough, greyish/pale, thick; diam. 3-4 mm.
51 C	rod shaped; spore; spaerical, terminal, swallen; gram +	circular, convex, edge slightly rough, pale, dry,thick,diam. 3-4 mm.
142 A	rod shaped; spore; ellipsoidal, central, swallen; gram +	circular,slightly flat, edge rough, pale/whitish, dry; 2-3 mm

Species: 23 A : *B. sphaericus* (new strain)
25 C : *B. pumilus*
51 C : *B. sphaericus* (new strain)
142 A : *B. cereus*

DISCUSSION

Many investigations have been conducted on the isolation of entomogenous microorganisms associated with insects of agricultural and medical importance. In Indonesia, these kind of investigations are still rare, although experts from WHO had initiated such investigations and successfully isolated *B.sphaericus* (assesion number 1593-4 and 1482-2).

Intensive investigations on the characteristics or atributes and the possibility of using *B.sphaericus* strain 1593 for mosquito control are now being carried out by many experts in different countries. According to Dr.H. de Barjac (1989, by letter communication), the sporeforming bacteria, *B.sphaericus* strain 23A and 51C reported in this paper, are different from the former *B.sphaericus* isolated from Indonesia, hence add to the collection of potential microbial agents for mosquito control.

In studies on the toxicity test of microbial agents as shown, for example, by many strains of *B. thuringiensis* and *B.sphaericus,* the toxicity varies considerably with a given insect species[6]. Several isolates in the present study were toxic to *C.quinquefasciatus.*

Crystallyferous strains of *B.thuringiensis* having highly preferential toxicity[12,6] and no toxicity[13,12,6] have been reported. Although toxicity tests have been done qualitatively, and which have only been tested

against *C.quinquefasciatus*, it is possible that our isolates, which showed no toxicity to the one species of the insect tested, have selected toxicity to other species or groups of insects. Hence, the screening of various strains of *Bacillus spp.* (including those of the four isolates) to other insect species is important in utilizing these microorganisms for microbial control.

The isolation of the four bacterial pathogens mentioned before could be regarded as a clue to the rich flora of bacterial pathogens in Indonesia. Isolation, identification, characterization, toxicity tests, and fermentation studies are now in progress. A continuing survey on bacterial pathogens is being considered as part of our future work.

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